Dexamethasome regulation of cyclin,cdk and Rb protein in L929.06 mouse fibroblast cells

博士論文

Dexamethasone regulation of cyclin, cdk and Rb protein in L929.06 mouse fibroblast cells
(Dexamethasone によるL929.06 マウス線維芽細胞の cyclin, cdk, Rb protein の制御)

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Abstract

Dexamethasone regulates the proliferation of L929.06 mouse fibroblast cells. Dexamethasone inhibited the hyperphospholylation of Rb protein which was thought to be required for cells to enter into S phase in the cell cycle. The inhibitory effect was reversible and the Rb protein was hyperphosphorylated 2hr after the removal of dexamethasone. Dexamethasone inhibited cyclin E-associated histone H1 kinase activity and cyclin D1 or cyclin D3-associated Rb protein kinase activity. Cyclin D1 and cyclin D3-associated kinase activities showed the similer kinetic changes with the phosphorylation status of Rb protein in the dexamethasone withdrawal experiment. The expression of cyclin E or cyclin D proteins was not changed by the dexamethasone treatment. These observations indicate that dexamethasone regulation of L929.06 mouse fibroblast cell proliferation does not involve inhibitions of the expression of cyclin or cdk proteins. The different mechanisms might be involved in the inhibitory effect of dexamethasone on the kinase activities in late G1 phase of the cell cycle to inhibit the proliferation of L929.06 mouse fibroblast cells.

Introduction

The proliferation of eukaryotic cells is regulated by a decision whether they remain in the cell cycle and replicate their chromosomal DNA or to withdrawal from the cell cycle which is made late in the first gap (G1) phase of the cell cycle (1,2) The G1-specific activities of holoenzymes are rate limiting for the passage through the point in late G1. Once a cell passes this critical point, it will complete the cell cycle even if mitogenic signals are removed (3).

The progression through the cell cycle is governed by a family of serine/threonine protein kinases that consist of a catalytic subunit of the cyclin-dependent kinases (cdks) and a regulatory subunit of the cyclins (4). In general, cyclins are expressed only at the specific points during the cell cycle. The expression of cyclins are regulated by rapid changes in the rate of synthesis and degradation of the cyclin gene products. On the other hand, the expression of cdks are consistent throughout the cell cycle (5-7).

The mammalian G1 cyclins are known to regulate the G1 phase of the cell cycle including three D-type cyclins (cyclin D1, D2 and D3) (14,15) and cyclin E (8). Cyclin E expression is periodic and peaks at the G1/S transition (9,10). It binds to cdk2 and activate the kinase (11,12, 25). Overexpression of cyclin E in fibroblasts were found to shorten the G1 interval, decreased the cell size and reduced the serum requirement for the G1/S transition (13). D-type cyclins generally appear earlier in G1 than cyclin E and they are differently expressed in various cell lines (16-18, 46). The D-type cyclins interact with cdk2, 4, 5 and 6 (19-22) depending on the cell line. The expression of cyclin D1 is crucial for G1 progression since the microinjection of cyclin D1 antisense plasmids or antibodies into serum-stimulated fibroblasts can prevent their entry into S phase (23-24).

Rb protein that is the product of the retinoblastoma susceptibility gene *Rb-1* (26) is the only known substrate for the activated cdk kinases (27) . Hyperphospholylation of Rb protein occurs during late G1 phase, and appear to be required for entry into S phase, whereas accumulation of underphosphorylated Rb protein prevents DNA synthesis (28-30).

Our laboratory has been interested in the dexamethasone regulation of proliferation of L929.06 mouse fibroblast cells. Previous observations in our laboratory show that dexamethasone inhibits cell proliferation of L929.06 cells reversibly (31). Flow cytometoric analysis and thymidine kinase expression assays indicate that dexamethasone arrests the L929.06 cells in the G1 phase of the cell cycle (31, 32). However, the mechanisms of how dexamethasone regulate the cell cycle of L929.06 cells is still unknown. The experiments described below were undertaken to test the hypothesis that dexamethasone regulates cyclin and cdk proteins leading to the inhibition of L929.06 cell proliferation.

Purpose

The purpose of this study was to examine the mechanism by which dexamethasone inhibits cell cycle progression in L929.06 mouse fibroblast cells.

Materials and methods

Cell culture

L929.06 cell line is a subclone of the mouse L cell (NCTC 929) selected for sensitivity to dexamethasone. L929.06 cells were grown in Dulbecco's modified Eagle's (DME) medium (Sigma Chemical Co., St Louis, MO) supplemented with 5% fetal bovine serum (FBS) (Gibco, Gaitheburg, MD) and subcultured by treatment with trypsin-EDTA (0.25% Trypsin, 1 mM EDTA-4Na) (Gibco).

Western blot analysis

L929.06 cells were plated at a density of 1.0 x 10^6 cells/100 mm dish in DME medium with 5% FBS. 3 days later, when in mid-logarithmic growth, the cells were treated with either vehicle (ethanol to final concentration, 0.1%) or dexamethasone (Sigma Chemical Co., St Louis, MO) (to a final concentration, 0.1 μM). The cells were washed and harvested with phosphate-buffered saline (PBS). The cells were resuspended in PIPA lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate (DOC), 200 μM Sodium Orthovanadate) containing 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml Pepstatin A and lysed by a 30 min incubation at 4°C. Protein concentrations in cell extracts were measured by the methods of Bradford. The protein samples were incubated at 95°C for 5 min with sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 10% β mercaptoethanol, 0.05% Bromophenol Blue) and separated by 6, 10 or 11% SDS-polyacrylamide gel electrophoresis (PAGE). The proteins were then transferred to nitrocellulose membrane(Schleicher and Schuell, Dassel, Germany) with a semidry blotting apparatus containing transfer buffer (250 mM Tris, 2 M Glycine, 20% methanol). The nitrocellulose membranes were blocked in TBST (10 mM Tris pH 7.4, 0.2 M NaCl, 0.05% Tween 20, 5% nonfat dry milk) for overnight. The nitrocellulose membranes were then incubated with primary antibody diluted with TBST for 1 to 3 hours, washed with TBST for 30 min and incubated with secondary antibody. We used the following primary antibodies: polyclonal antibody to

cyclin E, (dilution 1:500) (UBI, Lake Placid, NY), polyclonal antibody to cyclin D1, (1:500) (UBI), polyclonal antibody to cyclin D2 (1:500) (Santa Cruz, Delaware, CA), monoclonal antibody to cdk2 (1:5000) (Santa Cruz), polyclonal antibody to cdk4 (1:500) (Santa Cruz), polyclonal antibody to cdk6 (1:500) (Santa Cruz), monoclonal antibody to Rb protein (1: 250) (Pharmingen, San Diego, CA). Antiserum to the complete coding sequence of mouse cyclin D3 was prepared by immunizing rabbits with recombinant mouse cyclin D3 (46) and was used at 1:2000 dilution. The secondary antibodies were either goat antibodies to rabbit IgG-HRP (immunoglobulin G-horse radish peroxidase) or goat antibodies to mouse IgG-HRP. The detection system used was enhanced chemiluminescence (ECL, Amersham and Kodak XAR-5 film).

Immunoprecipitation

L929.06 cells were grown, treated and lysed as same protocol as shown in western blot analysis. Immunoprecipitation was performed by incubation of cell lysate with 1 μl of antibody specific to cyclin E, cyclin D1 or cyclin D3 for 1 hr on ice. Immunoprecipitates were collected by incubation with 10% of protein A-sepharose (Sigma Chemical Co.) for 2 hr.

Histone H1 kinase assay

The immune complexes with protein A-sepharose beads were washed three times with PIPA lysis buffer, then three times with reaction buffer (20 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$). The beads were resuspended in the 5 μl of kinase assay mixture (80 μM [γ-^{32}P] ATP (adenosine triphosphate) (Dupont), 2 μg histone H1 (Sigma), 2X reaction buffer) and incubated at 37°C for 30 min. The reaction was stopped by addition of 5 μl of 2X sample buffer. The samples were separated by electrophoresis on an 11% polyacrylamide gel. Histone H1 was visualized by Coomassie blue staining (40% methanol, 0.025% Coomassie blue (Sigma), 7.0% Acetic acid) and the gel was dried. Bands were detected by autoradiography of dried gels with XAR-5 films (Kodak). The signals were quantified by a Lynx densitometer (Applied Imaging).

Purification of GST-Rb protein

GST-Rb in p56 version of Rb (aa 394-928) cloned in *Sma* I site of pGST-2T was a gift from Dr. Alice Covanaugh. A transformant colony was inoculated with 50 ml of LB (100 μg/ml ampicillin, 30 μg/ml chloramphenical) at 37°C for overnight. The 50 ml was then inoculated with 500 ml for overnight. The fusion Rb protein was induced by adding 200 mM isopropyl-1-thio-β-D-galactoside (IPTG) (Sigma) to 0.2 mM and incubated at 37°C for 2 hr. The culture was spinned down and the pellet was kept at -70°C. The pellet was lysed in 20 ml of lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 5 mM DTT, 0.2% NP-40, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 10 μg/ml PMSF, 10 μg/ml Pepstatin A), sonicated three times, 30 sec, cooling in between. The sample was spinned down and supernatant was kept at -70°C. For the purification of the GST-Rb protein, we used the glutathione separose column (Pharmacica, Sweden). The column was washed with 20 ml of PBS and equilibrated with 6 ml PBS with 1% Triton X-100. The sample was applied to the column and left for 20 min. The column was washed with 10 ml of PBS twice. The bound GST-Rb protein was eluted with 10 ml of elution buffer (50 mM Tris-HCl pH 8.0, 5 mM glutathione) and collected 1 ml fractions. The protein concentration was measured with Bradford assay. The protein was aliquated and stored at -70°C.

Rb kinase assay

L929.06 cells were grown and treated as same protocol as shown in western blot analysis. The cells were collected with PBS. The cells were resuspended with IP buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1mM NaF, 0.1% Tween 20, 10% glycerol, 10 μg/ml aprotinin, 10 μg/ml PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 0.1 mM sodium orthovanadate, 1 mM DTT) and sonicated 15 sec three times, cooling in between on ice. The samples were immunoprecipitated following the protocol above. The immune complexes with protein A-sepharose beads were washes three times with IP buffer and twice with

washing buffer (50 mM HEPES pH7.5, 1 mM DTT). The beads were incubated with kinase mixture (1.5 μg purified GST-Rb protein, 1.0 μl [γ-^{32}P] ATP (6000 Ci/mmol), 6.0 μl 3X kinase buffer (150 mM HEPES pH7.5, 30 mM $MgCl_2$, 7.5 Mm EGTA, 30 mM β-glycerophosphate, 3 mM NaF, 3mM DTT, 0.3 mM sodium orthovanadate) at 30°C for 30 min. The reaction was stopped by addition of 2X sample buffer. The samples were incubated at 95 °C for 5 min and separated by electrophoresis on an 6% polyacrylamide gel. Rb protein was visualized by Coomassie blue staining and the gel was dried. Bands were detected by autoradiography of dried gel with XAR-5 film (Kodak). The signals were quantified by a Lynx densitometer (Applied Imaging).

Results

Dexamethasone effects on cyclin, cdk or Rb protein

Figure 1 shows the effects of dexamethasone on cyclin, cdk or Rb protein expressions in L929.06 cells. The L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. The cells were collected, lysed and western blot analysis was performed as described in Materials and methods. Dexamethasone treatment did not change the expression of cyclin D1, D2, D3 (Figure 1(a)), cdk2, 4 or 6 (Figure 1(c)). Two different molecular weights of cyclin E protein were detected and the upper band was partly migrated to the lower band by the dexamethasone treatment (Figure 1(b)). The total expression of cyclin E protein was not changed by the treatment. In Rb protein expression, two bands were detected. From the molecular weights, the upper band shows the hyperphosphorylated Rb protein and lower band shows the hypophosphorylate Rb protein. The Rb protein was dephosphorylated by the dexamethasone treatment (Figure 1(b)).

The protein expressions in the recovery study from the dexamethasone treatment

Dexamethasone was reported to arrest the cell cycle of L929.06 cells at the G1 phase. To examine the expression of cyclin, cdk or Rb proteins after recovering from

dexamethasone treatment, the dexamethasone withdrawal study was performed. L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. The cells were washed with DME medium and fed with fresh DME medium with 5% FBS without dexamethasone (time 0). The cells were collected, lysed and freezed at time 0, 2, 4, 8, 12 and 24 hr after removal of dexamethasone. Western blot analysis was performed as described in Materials and methods. In this study, cyclin D1 and cyclin D3 protein expressions showed higher levels at the beginning and reduced after 12 hr from the removal of dexamethasone (Figure 2). Hyperphosphorylated Rb protein increased at 2 hr and reduced at 8 hr (Figure 2). The expression of cyclin E or cdk proteins did not change in this dexamethasone withdrawal study (Figure 3).

The protein expressions in the recovery study from the serum starvation

To compare the effects of dexamethasone on the expression of cyclin or Rb protein with the serum starvation in L929.06 cells, the recovery study from the serum starvation was performed. L929.06 cells were cultured in DME medium with 0.25% FBS for 3 days. The cells were washed with DME medium and fed with fresh DME medium with 5% FBS (time 0). The cells were collected, lysed and freezed at time 0, 2, 4, 8, 12 and 24 hr after the addition of 5% FBS. Western blot was performed as described in Materials and methods. In this recovery study from serum starvation (Figure 4), the expressions of cyclin D1 and hyperphosphorylated Rb protein peaked at 12 hr after the addition of 5% FBS. The expression of cyclin D3 protein peaked at 4 hr and cyclin E protein did not change.

Dexamethasone effects on cyclin E-associated histone H1 kinase activity

To examine the kinase activity associated with cyclin E, cyclin E-associated histone H1 kinase activity assay was performed. The histone H1 was used as the substrate in this assay. L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. In the time course study, cells were washed and fed with fresh DME medium with 5% FBS as described above. The cells were collected and lysed at

the indicated time. The samples were immunoprecipitated with cyclin E antibody and histone H1 kinase assay was performed as described in Materials and methods. Figure 5 shows the results of the cyclin E-associated histone H1 kinase activity assay. Dexamethasone inhibited cyclin E-associated histone H1 kinase activity (Figure 5(a)). In the dexamethasone withdrawal study, the kinase activity started to increase at 4 hr after the removal of dexamethasone (Figure (b)).

Dexamethasone effects on cyclin D1 or cyclin D3-associated Rb kinase activity

To examine the kinase activity associated with cyclin D, cyclin D-associated Rb kinase activity assay was performed. In this study, the Rb protein (see Materials and methods for purification) was used as the substrate for the assay. L929.06 cells were treated and lysed with same protocol as described in the histone H1 kinase assay above. The samples were immunoprecipitated with cyclin D1 or D3 antibody. The Rb kinase assay was then performed as described in Materials and Methods. As shown in Figure 6(a), dexamethasone inhibited both cyclin D1 and D3-associated Rb kinase activities. The inhibition was grater in cyclin D3-associated kinase. In the dexamethasone withdrawal study, the kinase activity increase at 2 hr and reduced at 8 hr after removal of dexamethasone (Figure 6(b)(c)).

Discussion

Glucocorticoids play an important physiological and pharmacological role in regulating proliferation of fibroblastic cells (33). A number of investigators have shown that the proliferation of mouse L929 fibroblast cell is inhibited by low concentration (10^{-9} to 10^{-7} M) of glucocorticoids (34-35). The inhibitory effects of glucocorticoids in mouse fibroblasts reflect the clinical efficacy of such compounds as anti-inflammatory agents (35,38). Our laboratory has reported that dexamethasone inhibits the proliferation of L929.06 cells in culture (31). Incorporation of [^{3}H]thymidine is also inhibited by 70-90% within 24 hr after addition of 0.1 μM of dexamethasone (32). Since cyclin, cdk and

Rb proteins are though to be important in controlling cell cycle progression, we have undertaken the experiment described to determine if glucocorticoids regulate the expression of these proteins and if such regulation contributes to glucocorticoids-induced inhibition.

The first question we had was where in G1 phase of the cell cycle L929.06 cells were arrested by dexamethasone. To solve this question, dexamethasone withdrawal study and serum starvation study were performed (Figure 2, 3, and 4). Two bands of different molecular weight of Rb protein were detected in these experiments. Upper band showed the hyperphosphorylated Rb protein and lower band showed the hypophosphorylate Rb protein. Hypophosphorylated Rb protein can interact with the transcription factor E2F during G1 phase (39). This complex can bind to DNA and repress transcription of target gene, some of which are normally expressed in S phase (40, 41). Conversely, hyperphospholylation of Rb protein prevents its interaction with E2F, releasing it from an inhibitory constraint, enabling it to promote gene expression and then cells can enter into S phase (50). So the phosphorylation of Rb protein occurs just before the G1/S transition. In the dexamethasone withdrawal study, the expression of hyperphosphorylated Rb protein peaked at 2 hr after the removal of dexamethasone. On the other hand, in the serum starvation study, the hyperphosphorylated Rb protein peaked at 12 hr after the addition of the serum. So there is 10 hr interval between the points where these cells were arrested in these two experiments. From these results, dexamethasone was considered to arrest the cell cycle of L929.06 mouse fibroblast cells at late G1 phase.

The second question was what kinds of kinases were contributed to the phosphorylation of Rb protein at this late G1 phase of the cell cycle. To examine which cyclins are associated with the kinases that are related to the phosphorylation of Rb protein, cyclin D1, D3 or E-associated kinase assay was performed. In the cyclin E-associated histone H1 kinase assay, dexamethasone inhibited the kinase activity (Figure 5). In the dexamethasone withdrawal study, the cyclin E-associated kinase activity was started to increase at 4 hr after the removal of dexamethasone. Since the phosphorylation

of Rb protein was started 2 hr after the removal of dexamethasone (Figure 3), cyclin E-associated kinase activity started to increase later than this period. Both cyclin D1 and cyclin D3-associated kinase activities were inhibited by dexamethasone treatment (Figure 6). In the dexamethasone withdrawal study, both cyclin D1 and cyclin D3-associated kinase activities increased at 2 hr and decreased at 8 hr after the removal of dexamethasone that is the similar changing with the phosphorylation status of Rb protein (Figure 2). From these results, the kinases that are associated with cyclin D1 or/and cyclin D3 were thought to regulate the phosphorylation of Rb protein.

The third question was how dexamethasone regulates these these kinase activities that phosphorylate Rb protein in the G1 phase of the cell cycle. Two main mechanisms have been proposed in regulation of the kinase activity in G1 phase. The first one is the amount of cyclins that bind to cdk proteins regulates kinase activities. Each cyclin is expressed at the specific point during the cell cycle. The expression of cyclins is regulated by the rapid changes in the rates of synthesis and degradation. On the other hand, the expression of cdk proteins is generally constant throughout the cell cycle. The kinase activity is regulated by the amount of cyclins that bind to cdk protein. The experiment described in Figure 1 showed that dexamethasone did not change the expression of cyclin E or cyclin D. Dexamethasone only changed the status of cyclin E protein. One of two bands of cyclin E was migrated to the other by the dexamethasone treatment. Cyclin E is reported to have several phosphorylation sites (10). Dexamethasone may change the phosphorylation status of cyclin E protein, though further study is needed to prove it. From these data, the kinase activity might not be regulated by the amount of cyclins that bind to cdk proteins. The other mechanism is the existence of kinase inhibitors. Three cyclin kinase inhibitors, $p16^{Ink4}$, p21, $p27^{Kip1}$ have been identified in vertebrates (37). The p21 is a 21 kD protein that binds to both cyclin D/cdk4 and cyclin E/cdk2 complexes (42-46). Its expression is regulated by p53 and plays an important role in G1 arrest after DNA damage (42, 47). The p16 is a 16 kD protein that binds to cdk4 protein and it causes the dissociation of the cyclin D/cdk4 complexes (48). The p27 is a 27 kD protein that binds to cyclin E/cdk2 or cyclin D/cdk4

complex and inhibits cyclin E/cdk2 (38). In the experiment, dexamethasone decreased the kinase activities despite no change in the cyclin or cdk protein levels. So it is hypothesized that the kinase inhibitors might be involved in the inhibitory effect of dexamethasone on the cell cycle progression.

Conclusion

In this paper, we have shown that dexamethasone inhibits kinase activities associated with cyclins in late G1 phase of cell cycle leading the inhibition of cell proliferation in L929.06 mouse fibroblast cells. Further studies are needed to examine the mechanisms of the inhibitory effect of dexamethasone on the proliferation of the cells.

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Legends

Figure 1: Effects of dexamethasone on cyclin, cdk or Rb protein expression in L929.06 mouse fibroblast cells. The L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. The cells were collected, lysed and western blot analysis was performed as described in Materials and methods. These autoradiograms are representative of three separate experiments.

Figure 2: The protein expressions of cyclins or Rb protein in the recovery study from the dexamethasone treatment. L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. The cells were washed with DME medium and fed with fresh DME medium with 5% FBS without dexamethasone (time 0). The cells were collected, lysed and freezed at time 0, 2, 4, 8, 12 and 24 hr after removal of dexamethasone. (**a**) Western blot analysis was performed as described in Materials and methods. These autoradiograms are representative of two separate experiments. (**b**) The levels of proteins were quantitated by densitometric scanning of the autoradiograms.

Figure 3: The protein expressions of cdk proteins in the recovery study from the dexamethasone treatment. L929.06 cells were treated and collected as same as described in Figure 2. (**a**) Western blot analysis was performed as described in Materials and methods. These autoradiograms are representative of two separate experiments. (**b**) The levels of proteins were quantitated by densitometric scanning of the autoradiograms.

Figure 4: The protein expressions of cyclins or Rb protein in the recovery study from the serum starvation. L929.06 cells were cultured in DME medium with 0.25% FBS for 3 days. The cells were washed with DME medium and fed with fresh DME medium with 5% FBS (time 0). The cells were collected, lysed and freezed at time 0, 2, 4, 8, 12 and 24 hr after the addition of 5% FBS. (**a**) Western blot was performed as described

in Materials and methods. These autoradiograms are representative of two separate experiments. (**b, c**) The levels of proteins were quantitated by densitometric scanning of the autoradiograms.

Figure 5: Dexamethasone effects on cyclin E-associated histone H1 kinase activity. (**a,b**) L929.06 cells were treated with either vehicle or 0.1 μM of dexamethasone for 24 hr. In the time course study, cells were washed and fed with fresh DME medium with 5% FBS as described in Figure 2. The cells were collected and lysed at the indicated time. The samples were immunoprecipitated with cyclin E antibody and histone H1 kinase assay was performed as described in Materials and methods. These autoradiograms are representative of two separate experiments.(**c**) The levels of kinase activities were quantitated by densitometric scanning of the autoradiograms.

Figure 6: Dexamethasone effects on cyclin D1 or cyclin D3-associated Rb kinase activity. (**a,b**) L929.06 cells were treated and lysed with same protocol as described in the histone H1 kinase assay in Figure 5. The samples were immunoprecipitated with cyclin D1 or D3 antibody. The Rb kinase assay was then performed as described in Materials and Methods. These autoradiograms are representative of two separate experiments.(**c**) The levels of kinase activities were quantitated by densitometric scanning of the autoradiograms.

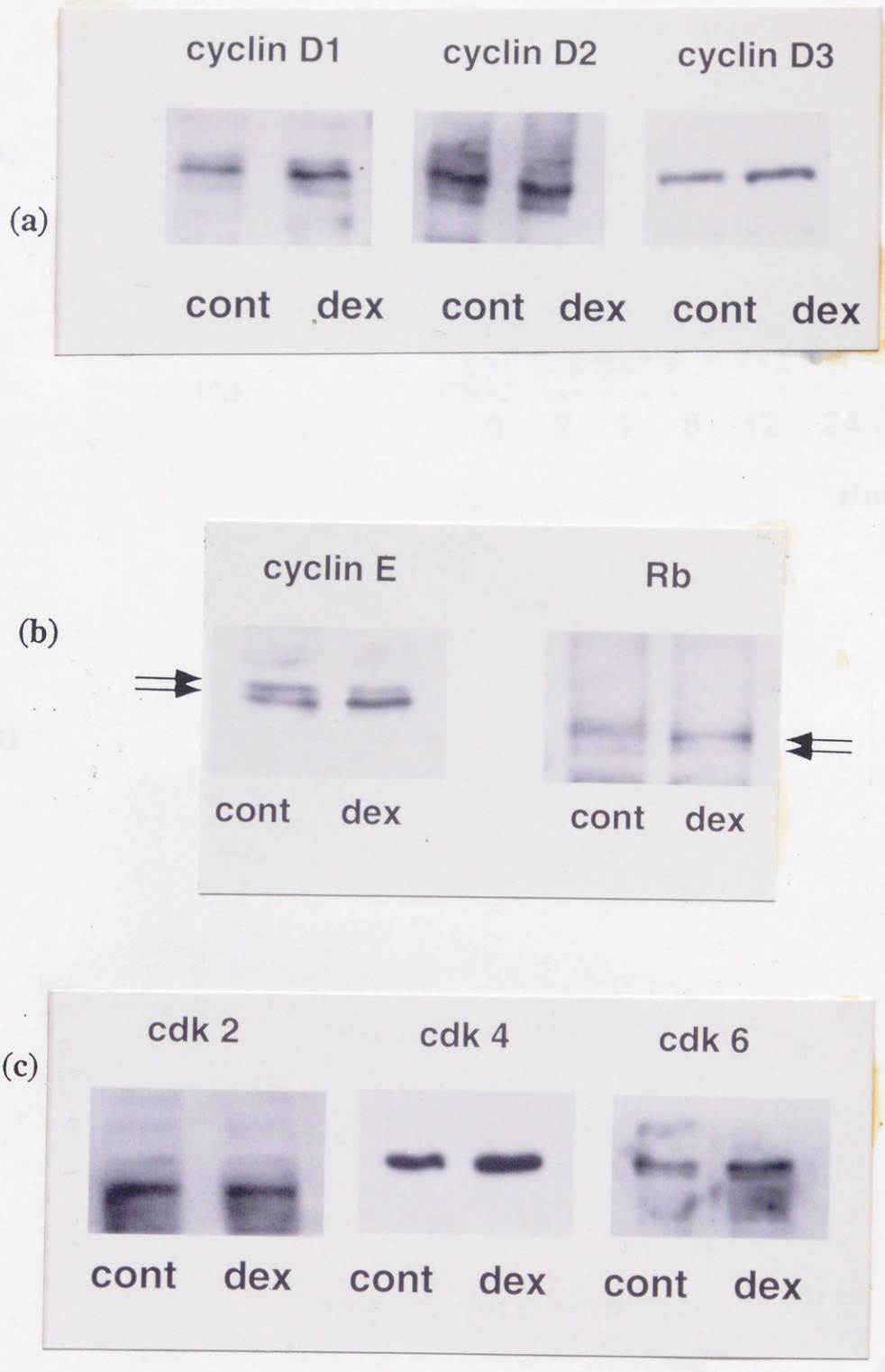
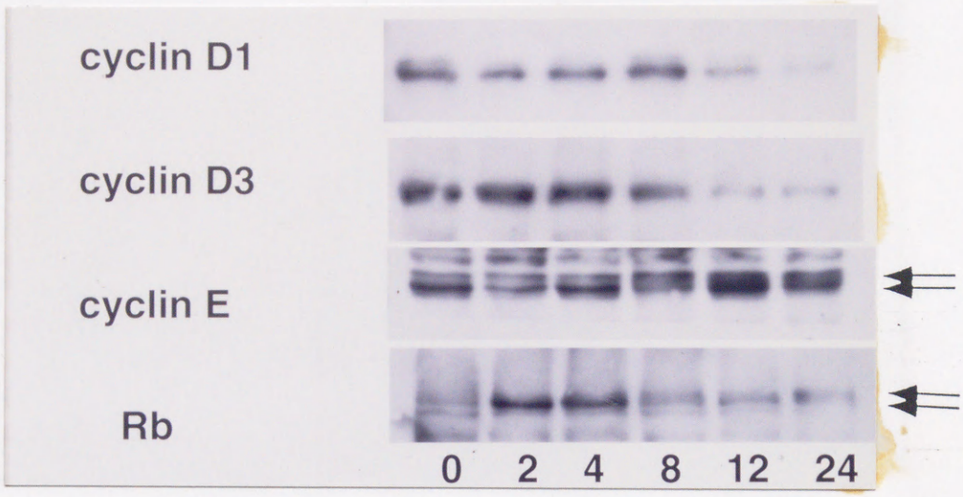

(a)
cyclin D1
cyclin D2
cyclin D3
cont
dex
cont
dex
cont
dex
(b)
cyclin E
Rb
cont
dex
cont
dex
(c)
cdk 2
cdk 4
cdk 6
cont
dex
cont
dex
cont
dex

Figure 1

(a)


cyclin D1
cyclin D3
cyclin E
Rb
0 2 4 8 12 24
Hours

(b)

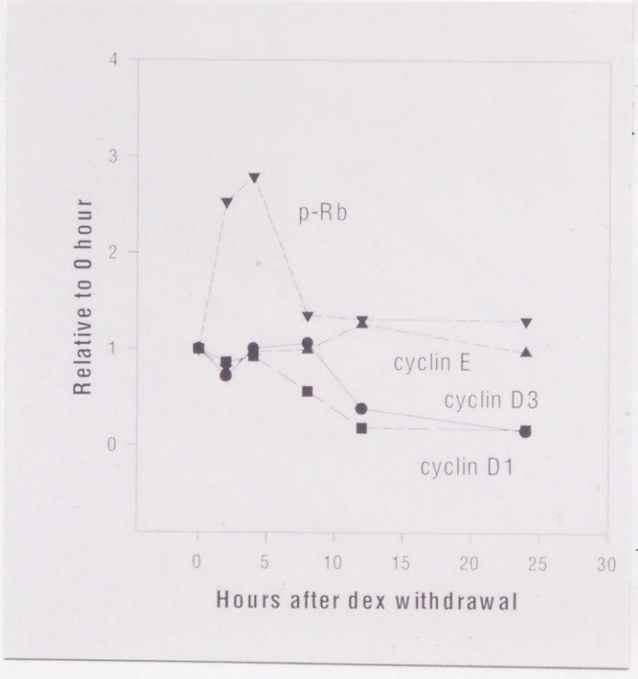
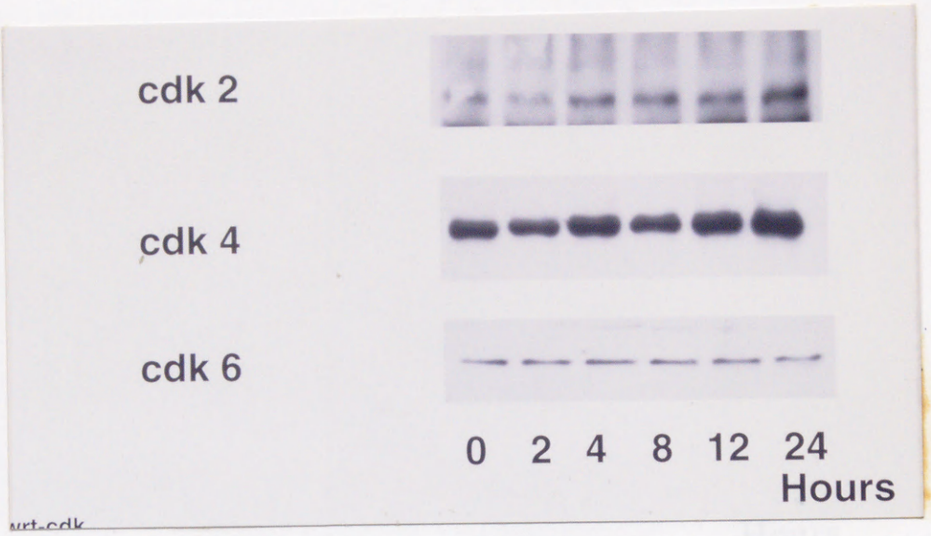

Relative to 0 hour
4
3
2
1
0
p-Rb
cyclin E
cyclin D3
cyclin D1
0 5 10 15 20 25 30
Hours after dex withdrawal

Figure 2

(a)


cdk 2
cdk 4
cdk 6
0 2 4 8 12 24
Hours

(b)

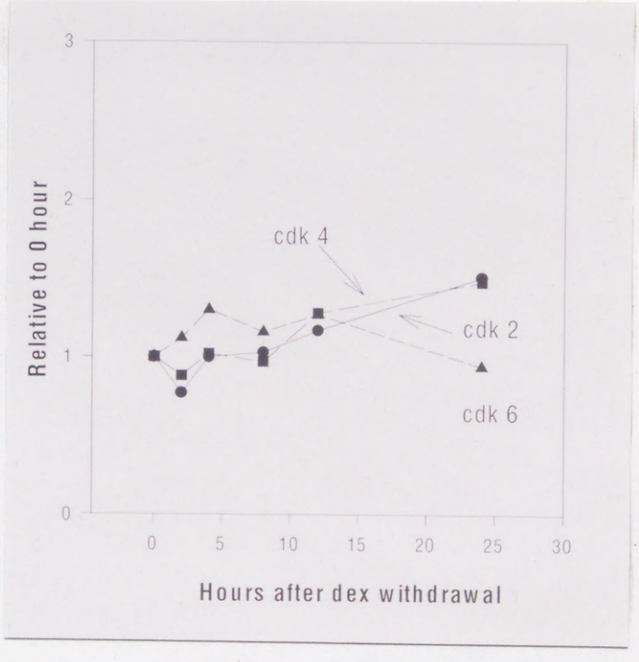
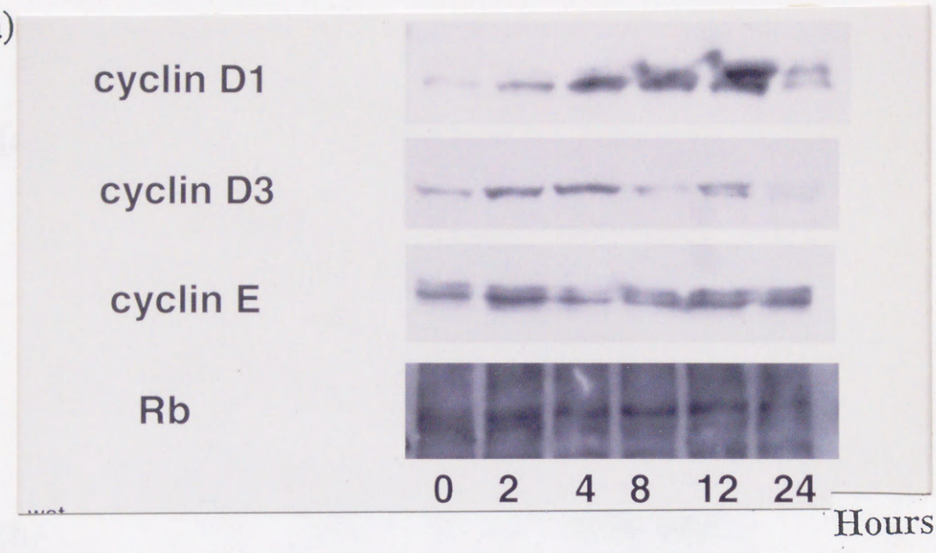
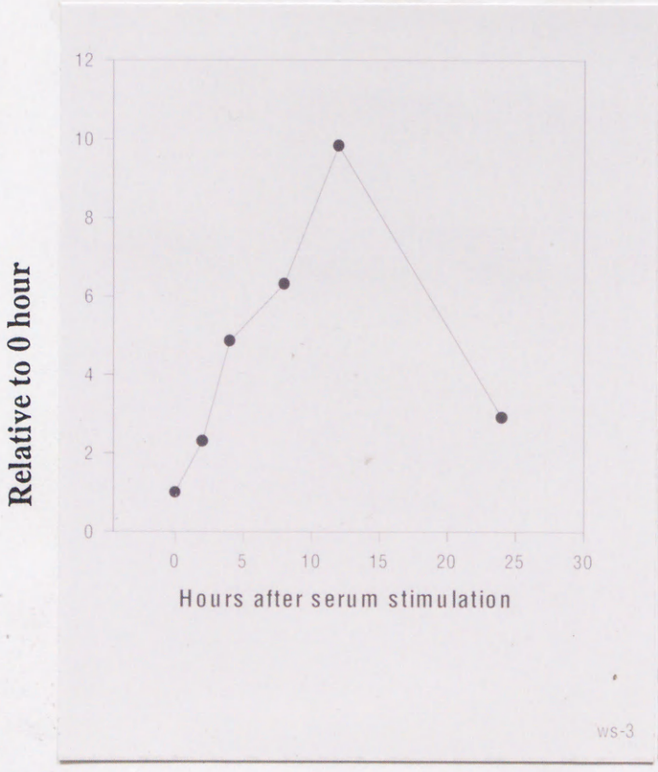

Relative to 0 hour
3
2
1
0
cdk 4
cdk 2
cdk 6
0 5 10 15 20 25 30
Hours after dex withdrawal

Figure 3

(a)


cyclin D1
cyclin D3
cyclin E
Rb
0 2 4 8 12 24
Hours

(b)


Relative to 0 hour
0
2
4
6
8
10
12
0 5 10 15 20 25 30
Hours after serum stimulation
ws-3

(c)

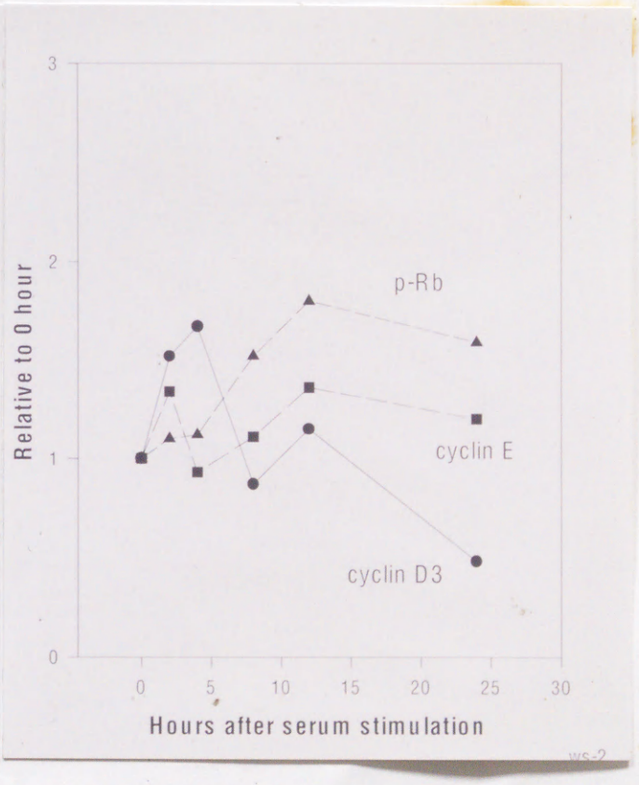
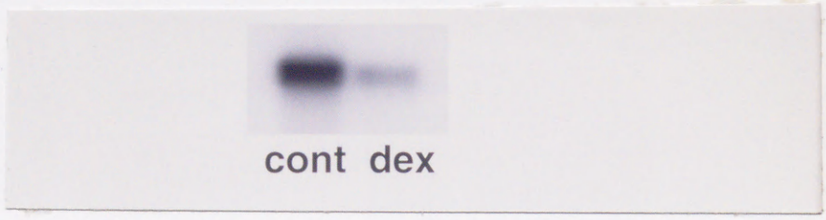
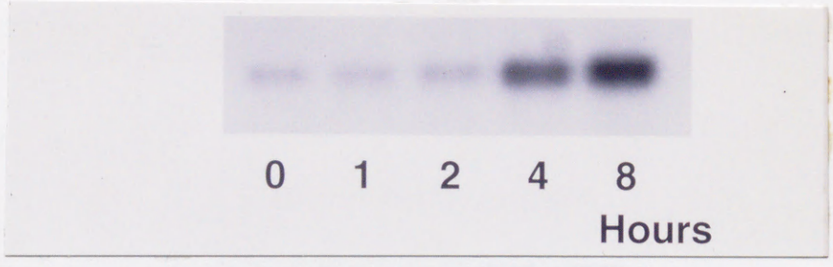

Relative to 0 hour
0
1
2
3
p-Rb
cyclin E
cyclin D3
0 5 10 15 20 25 30
Hours after serum stimulation

Figure 4

(a)


cont dex

(b)


0 1 2 4 8
Hours

(c)

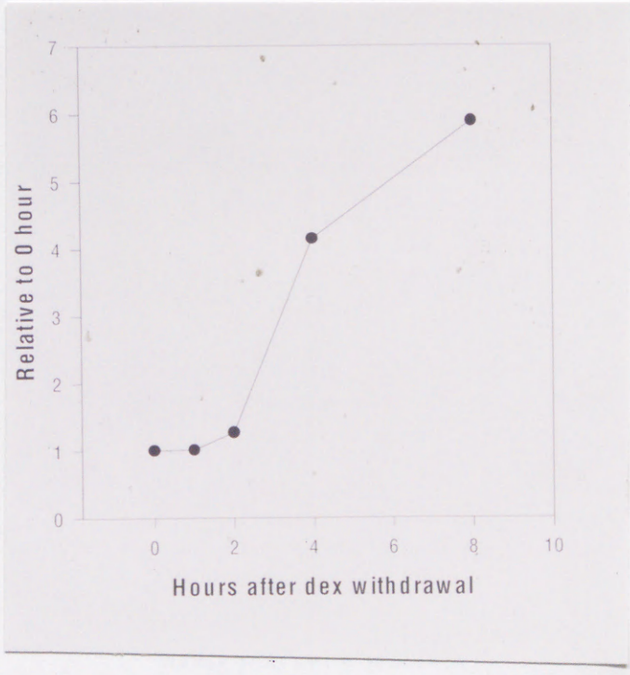
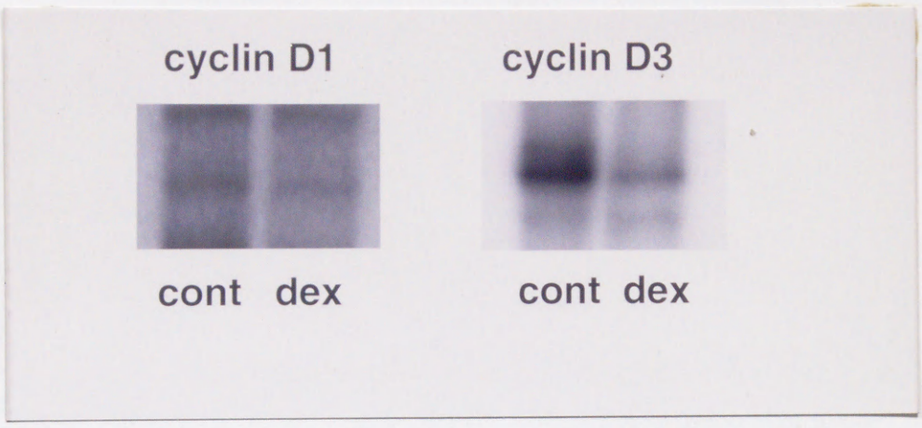
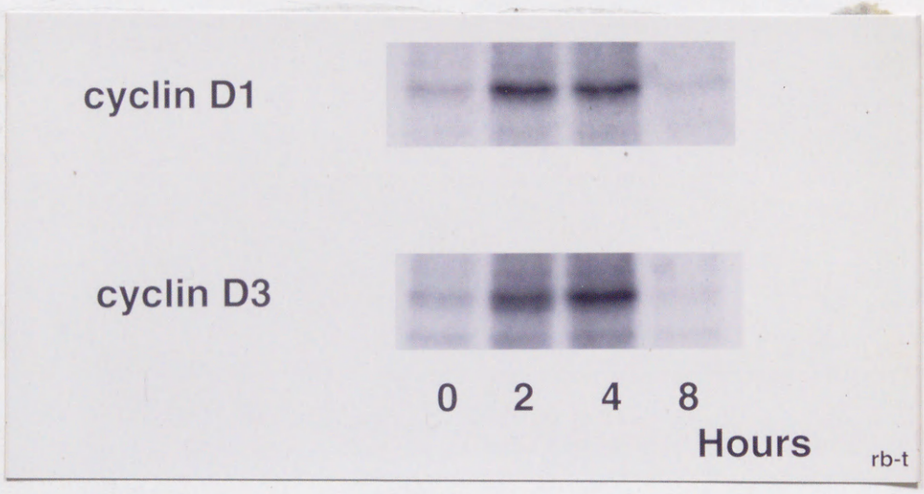

Relative to 0 hour
0
1
2
3
4
5
6
7
0
2
4
6
8
10
Hours after dex withdrawal

Figure 5

(a)


cyclin D1
cyclin D3
cont dex
cont dex

(b)


cyclin D1
cyclin D3
0 2 4 8
Hours
rb-t

(c)

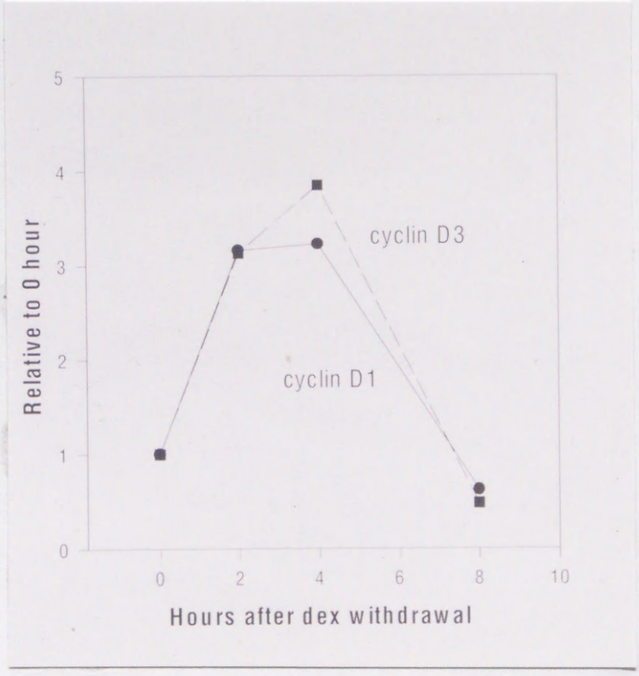
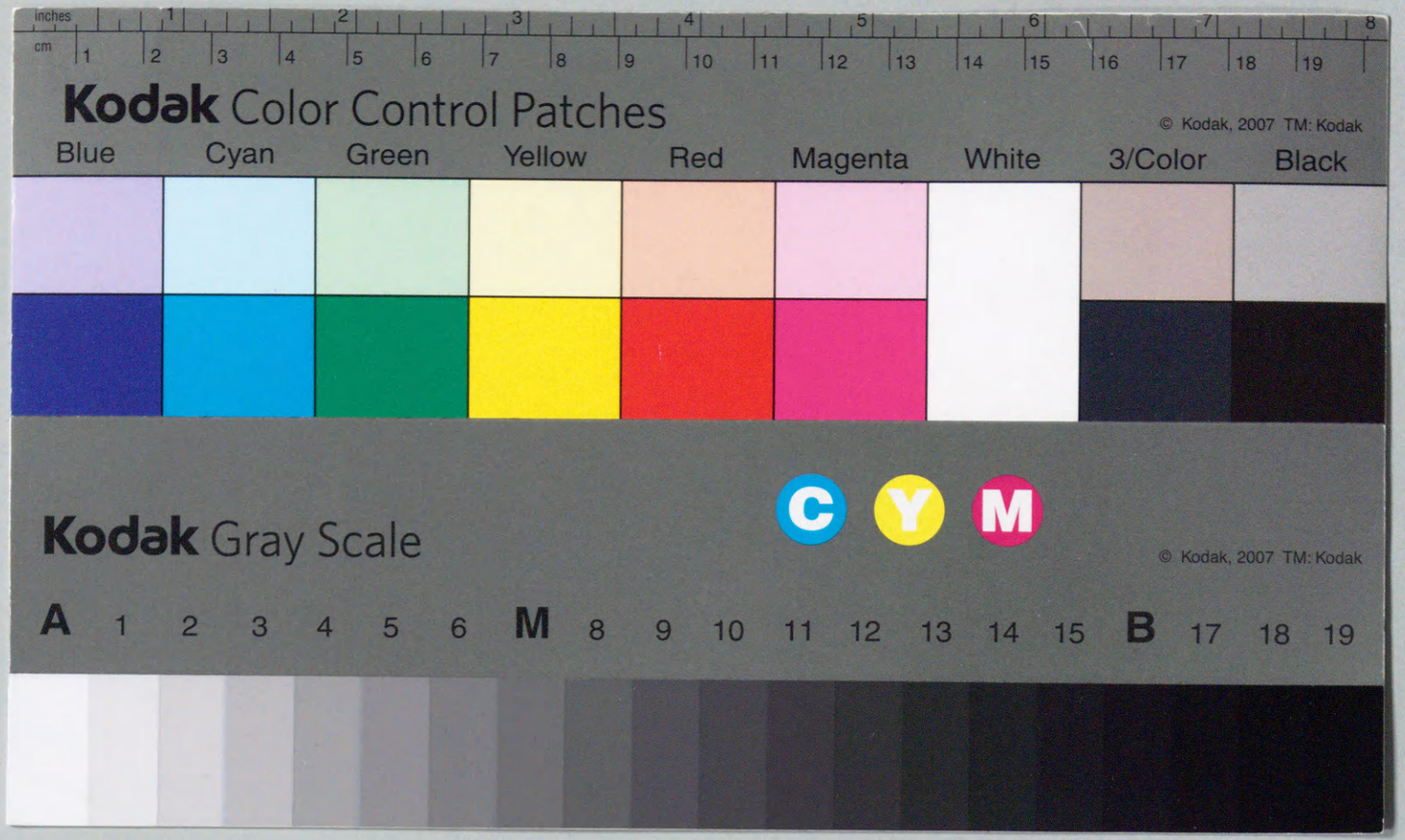

Relative to 0 hour
cyclin D3
cyclin D1
0 2 4 6 8 10
Hours after dex withdrawal

Figure 6


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19